Sporulation of *Streptomyces griseus* in Submerged Culture

KATHLEEN E. KENDRICK*† AND JERALD C. ENSIGN

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received 8 November 1982/Accepted 12 April 1983

A wild-type strain of *Streptomyces griseus* forms spores both on solid media (aerial spores) and in liquid culture (submerged spores). Both spore types are highly resistant to sonication, but only aerial spores are resistant to lysozyme digestion. Electron micrographs suggest that lysozyme sensitivity may result from the thinner walls of the submerged spores. Studies of the life cycle indicate that neither streptomycin excretion nor extracellular protease activity is required for sporulation; the analysis of mutants, however, suggests that antibiotic production may be correlated with the ability to sporulate. A method was devised to induce the rapid sporulation of *S. griseus* in a submerged culture. This method, which depends on nutrient deprivation, was used to determine that either ammonia or phosphate starvation can trigger sporulation and that the enzyme glutamine synthetase may be useful as a sporulation marker after phosphate deprivation.

The life cycle of streptomycetes is unique among bacteria. Under appropriate culture conditions, a single spore germinates, grows vegetatively as a substrate (primary) mycelium, and ultimately segments into chains of spores. The developing spores are typically enveloped by a sheath (3). The thick-walled spores are physiologically dormant when mature and are relatively resistant to lytic enzymes, low temperature, and osmotic extremes (3).

The sporulation process has been well documented with respect to the ultrastructure of cultures grown on solid media (5, 9, 17, 22). Physiological studies have been hampered by the inability to obtain uniform sporulation of the organism in a liquid culture. We have observed, however, that some streptomycetes can be induced to sporulate in a liquid culture when critical nutritional and environmental conditions are met. In this paper, we report the results of experiments conducted with a wild-type strain of *Streptomyces griseus*, which sporulates well when cultivated in liquid media. A method for the induction of rapid sporulation is described and used to characterize the physiology of sporulation with respect to nutritional requirements, enzyme behavior, and time course of differentiation.

METHODS

**Organisms and growth conditions.** Table 1 lists the strains used in this study. The strains were maintained on slants of medium A composed of casein hydrolysate (1 g/liter), yeast extract (5 g/liter), NaCl (5 g/liter), glucose (5 g/liter), and agar (15 g/liter), buffered with 10 mM 3-(N-morpholino)-propanesulfonic acid (MOPS; Sigma Chemical Co., St. Louis, Mo.-KOH, pH 7.2). The solid defined minimal medium B contained ferric citrate (0.05 g/liter), 1 mM Na<sub>2</sub>K<sub>2</sub>PO<sub>4</sub> (pH 7.2), 2 mM MgCl<sub>2</sub>, 0.03 mM CaCl<sub>2</sub>, 0.5 mM K<sub>2</sub>SO<sub>4</sub>, 30 mM MOPS-KOH (pH 7.2), 10 mM glucose, 1 mM L-asparagine, agar (15 g/liter), and trace salts (1 ml/liter). Medium C, the basal medium that was used for liquid cultures, contained 0.1 mM FeCl<sub>3</sub> (prepared at 10 mM in 50 mM sodium nitrilotriacetate, pH 7), 0.5 mM K<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 0.03 mM CaCl<sub>2</sub>, and trace salts (1 ml/liter). In most experiments, medium C was buffered with 50 mM Na<sub>2</sub>K<sub>2</sub>PO<sub>4</sub> pH 7.2. In media in which the phosphate concentration was at or below 1 mM, 50 mM MOPS-KOH (pH 7.2) or 50 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-KOH (pH 7.2) was used as the buffer. Trace salts contained 0.6 mM ZnCl<sub>2</sub>, 0.12 mM CuCl<sub>2</sub>, 0.10 mM MnCl<sub>2</sub>, 0.05 mM Na<sub>2</sub>MoO<sub>4</sub>, and 0.016 mM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>. Filter-sterilized carbon and nitrogen sources were added to autoclaved basal media. A 100-fold-concentrated solution of MgCl<sub>2</sub> and CaCl<sub>2</sub> was added to the remainder of the medium after separate autoclaving. All liquid cultures contained a coiled spring and 5% polyethylene glycol (molecular weight, 6,000; added as a 10% [wt/vol] solution after germination had occurred) to improve the dispersion of mycelia (10).

When auxotrophs were grown, serine was provided at 4 mM, and tryptophan and nicotinamide were provided at 0.1 mM, as required. Thiosstrepton (E. R. Squibb & Sons, Princeton, N.J.) was included at 1 μg/ml, rifampin (Sigma) was included at 10 μg/ml, and neomycin sulfate (Pfizer, Inc., New York, N.Y.) was included at 20 μg/ml in cultures of the appropriate drug-resistant mutants. The supplements were filter sterilized as concentrated stock solutions.

In most cases, liquid cultures were inoculated with spore suspensions harvested from medium B by the method of Hirsch and Ensign (7), except that the
spores were suspended and stored in water. The spores were activated by heating in 50 mM Trizma base (Sigma; pH 9.5) at 45°C for 15 min. The activated spores were collected by centrifugation at room temperature, suspended in water, and used as inoculum at a final concentration of 5 × 10^6 spores per ml. As a result of this treatment, more than 90% of the spores formed germ tubes by 5 h in complex medium or by 8 h in glucose-ammonia minimal medium. For those experiments involving nonsporulating strains, mycelial fragments served as the inoculum. Solid media were inoculated with either spores or mycelia. In most experiments, the cultures were incubated at 33°C. The cultures that were being tested for the production of streptomycin were incubated at 26°C, since in this hands this antibiotic was not produced at temperatures in excess of 28°C. Liquid cultures were shaken at 250 rpm in gyratory shakers.

Comparison of spore properties. The organisms were grown in medium C containing 20 mM glucose, 1 mM NH₄Cl, 50 mM phosphate buffer, and casein hydrolysate (vitamin and salt free [1 g/liter]; ICN-Nutritional Biochemicals, Cleveland), designated medium C+CAA, or on medium C+CAA solidified with agar (15 g/liter). The spores were harvested after incubation at 33°C for 7 days. Aerial spores (from solid cultures) were harvested by the glass bead technique (7); submerged spores (from liquid cultures) were harvested by centrifugation. Microscopic observation revealed that the liquid culture consisted of more than 95% spores; therefore, no precautions were needed to separate the spores from the mycelia. These spore preparations were washed twice in 1 M KCl to remove exocellular proteases (10), followed by four washes in distilled water. The suspensions were used immediately for physiological measurements; samples were lyophilized for electron microscopy.

Mycelia were obtained by harvesting cultures growing exponentially in medium C+CAA. All steps were carried out at temperatures higher than 33°C, and the mycelia were lysed when held at 4°C for even short lengths of time. The culture was washed twice in 50 mM K-PO₄ (pH 7.2) and used immediately.

Germination was measured by inoculating a heat-activated aqueous spore suspension in 50 mM K-PO₄ buffer (pH 7.2) containing 1.0 g of yeast extract per liter; after 4 h, the percentage of spores that had formed germ tubes was counted by examination with a phase-contrast microscope.

Endogenous respiration was measured by adding concentrated spores or mycelia to 3.0 ml of 50 mM K-PO₄ buffer (pH 7.2), equilibrated to 33°C. An oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio), was used to measure oxygen uptake. The results are given as the oxygen quotient (microliters of O₂ per hour per milligram [dry weight]).

Trehalase was determined in aqueous extracts of spores or mycelia prepared by boiling for 30 min. To 0.3 ml of extraction fluid was added 0.6 ml of 100 mM imidazole-hydrochloride (pH 7.3) and 0.1 ml of a crude preparation of trehalase from an extract of a soil bacterium that had been isolated by enrichment culture. This amount of trehalase was sufficient to effect complete hydrolysis of 1 mM trehalose under the reaction conditions used and did not hydrolyze sucrose, cellobiose, glycogen, lactose, or maltose. The mixture was incubated for 1 h at 36°C, and the reaction was stopped by boiling for 3 min. One half of this reaction mixture was combined with 0.5 ml of Statzyme reagent (Worthington Diagnostics, Freehold, N.J.; prepared at twice the concentration suggested by the manufacturer), and the absorbance was read at 500 nm.

Sonic resistance was assayed by subjecting spores or mycelia to 10 min of sonication, at 50% pulse, at the highest output that did not cause cavitation (Branson Sonifier, model 350). The spores were held at 4°C throughout the procedure, whereas the mycelia were kept at 20°C. Samples were removed, diluted, and plated on medium A for viable counts. Lysozyme resistance was assayed by measuring the percent survivors after incubation at 37°C for 1 h in 20 μg of lysozyme per ml in 20 mM TES buffer, pH 7.2.

For transmission electron microscopy, spore samples were fixed in 2.5% (vol/vol) glutaraldehyde–7 mM sodium cacodylate (pH 7.3) for 1 h at room temperature. The spores were homogeneously suspended in 15 g of Ionagar (Oxoid Ltd., London, England) per liter, and small agar cubes were postfixed in 0.85% (wt/vol) osmium tetroxide in 7 mM sodium cacodylate, pH 7.3. Fixed spores were dehydrated in ethanol and embedded in resin. Thin sections were stained in uranyl acetate–lead citrate and viewed with a Hitachi HU11E microscope.

Two-dimensional polycrystalline gel electrophoresis was carried out by the method of Roberts et al. (18). A spore suspension in lysis buffer (18) was combined with 5-μm-diameter glass beads (Ultrasonics, Plainview, N.Y.), at a ratio of 2 ml of spores per g of beads, in a dental amalgamator capsule (Caulk Vari-Mei II-M; L. D. Caulk Co., Milford, Del.). The chilled capsule was shaken at 4°C in the amalgamator for a total of 6 min, using 30-s bursts of the highest power separated by 30 s of cooling. Protein analysis (6) indicated that 87% of the total soluble spore protein was released by this method.

Quantification of sporulation. Because spores, but not mycelia, were resistant to sonic disruption, we measured sporulation efficiency (SE) as (colony-forming units per milliliter after 4 min of sonication)/(colony-forming units per milliliter in the absence of sonication). This value represents the fraction of the viable population that is spores. For some experiments, the sonic-resistant units per milliliter, the numerator of the above equation, are reported below.

Enzyme assays. Samples of culture were harvested by centrifugation and washed twice in 50 mM imidazole-hydrochloride, pH 7.3. The cells were suspended in 0.05 times the sample volume of imidazole buffer and disrupted with the dental amalgamator as described above. Cell debris was removed by centrifugation at 4°C, 15,000 × g, for 20 min. The supernatant fluid was used as the extract for all of the assays except the protease, for which the culture fluid was used. Specific activities are expressed as micromoles (substrate converted to product) per minute per milligram of protein.

Glucose-6-phosphate dehydrogenase was assayed by combining 0.1 ml of the extract with 100 mM imidazole-hydrochloride (pH 7.3) containing 2 mM glucose 6-phosphate (Sigma), 0.13 mM NADP (Sigma), and 1 mM MgCl₂ in a final volume of 3 ml. The mixture was incubated at 33°C for 1 h. The change in absorbance at 340 nm was compared with the extinc-
tion coefficient for NADPH of 6,220 liters mol$^{-1}$ cm$^{-1}$ (20).

Alkaline phosphatase activity was assayed with the substrate $p$-nitrophenylphosphate (Sigma). In a final volume of 3 ml, 100 mM Tris-hydrochloride (pH 8.5), 1 mM MgCl$_2$, and 2 mM $p$-nitrophenylphosphate were mixed with 20 to 100 $\mu$L of cell-free extract. The reaction was monitored spectrophotometrically at 420 nm, at which wavelength the extinction coefficient of $p$-nitrophenol is 13,200 liters mol$^{-1}$ cm$^{-1}$ (16).

We determined glutamine synthetase by the method of Kustu and McKereghan (13), using 20 to 50 $\mu$L of cell-free extract and incubating the reaction at 33°C. Enzyme activity was measured at pH 7.3 in the presence of manganese.

Extracellular protease was measured by the method of Ginther (4), with Azocasein (Sigma) as the substrate.

**Nutrient starvation.** Medium C containing 20 mM glucose, 20 mM NH$_4$Cl, 50 mM Na$_2$PO$_4$ (pH 7.2), and 10 g of Bacto-Peptone (Difco Laboratories, Detroit, Mich.) per liter was inoculated with spores or mycelial fragments. When the culture had grown to 150 to 200 Klett units (Klett-Summoner photocolorimeter, filter no. 42), the mycelia were harvested by centrifugation. All steps were performed at room temperature. The pellet was washed once with starvation medium identical to that described above but lacking peptone and either glucose, ammonia, or phosphate. Medium lacking phosphate was buffered with either 50 mM MOPS-KOH (pH 7.2) or 50 mM TES-KOH (pH 7.2). The washed cells were inoculated to a Klett value of approximately 70, into starvation medium pre-equilibrated to 33°C. This time of transfer was taken as 0 h.

Ammonia was measured as described by Kendrick and Wheelis (10). Glucose was assayed with the Worthington Statzyme reagent. Inorganic phosphate was measured colorimetrically, by the procedure of Leloir and Cardini (15). In these assays, MOPS buffer was substituted for TES in the medium because the latter interfered with the ammonia assay, and acid was used instead of polyethylene glycol, which interfered with the inorganic phosphate reaction. Neither of these substitutions had any effect on the time course of sporulation.

**Mutagenesis.** All steps were carried out in the dark. A suspension of spores in water was irradiated with mixing under a germicidal lamp having a median output at 260 nm. The spores were irradiated to a survival of 1%, diluted, plated on medium A, and incubated at room temperature in the dark for 7 days. Auxotrophs were detected by their inability to grow when replicated to medium B. Strain SKK817 was detected by the absence of white, powdery growth on medium A. All of the drug-resistant mutants arose spontaneously.

**Streptomycin bioassay and cross-feeding experiments.** *Streptomyces viridochromogenes* NRRL B-1511 and a spontaneously derived streptomycin-resistant mutant (resistant to 100 $\mu$g of streptomycin sulfate per ml) were the indicator organisms for the streptomycin bioassay by the agar overlay technique. Streptomycin assay bottom agar contained 15 g of beef extract per liter, 3 g of yeast extract per liter, 6 g of peptone per liter, and 15 g of agar per liter, adjusted to pH 7.9. Top agar included streptomycin assay bottom agar and 7 g of agar per liter. To measure production of the antibiotic by the test organism grown in the liquid culture, we overlaid the plates with top agar containing spores of the indicator strain and filled 6-mm wells with the culture filtrate; to assay drug production by test organisms grown on agar surfaces, we placed agar culture plugs of the test producer organism on the surface of the basal agar and overlaid the seeded top agar. The zones of inhibition were measured after overnight incubation at 33°C.

Sensitivity to streptomycin was assayed by the well-overlay method described above. Spores or mycelial fragments of the test organism were included in the top agar, and dilutions of streptomycin sulfate were added to the wells.

A similar method was used to assay for the excretion of a substance that stimulated sporulation. Agar plugs, removed at daily intervals from a confluent culture of strain B-2682 growing on medium B, were placed on the surface of a fresh medium B plate. The plate was overlaid with mycelial fragments of strain SKK817 suspended in 7 g of agar per liter. The growth of strain SKK817 in zones surrounding each plug was observed during incubation at 33°C for 6 days.

**RESULTS**

**Comparison of aerial and submerged spores.** We initially observed that *S. griseus* B-2682 sporulated after growth in a variety of media. Phase-contrast photomicrographs (Fig. 1) of the organism growing in medium C+C-AA show that a 24-h culture consisted of vegetative mycelia. The first indication of sporulation, characterized by swollen mycelial termini, occurred at approximately 36 h. Free spores were microscopically visible 45 h after inoculation. By 72 h, the entire culture consisted of spores.

The spores produced in the liquid culture (submerged spores) were compared with those produced on the solid medium (aerial spores). Their properties are listed in Table 2. In several respects, submerged spores resembled aerial spores and were distinct from vegetative mycelia. Although each type of spore was resistant to sonic disruption, submerged spores were as sensitive to lysozyme treatment as were mycelia, whereas aerial spores were resistant.

To explore the possibility that this major difference between submerged and aerial spores was due to the sheath (3), we examined both spore types by electron microscopy. An examination of thin sections by transmission electron microscopy indicated that the sheath was rarely present on aerial spores, whereas no ensheathed submerged spore specimens were seen. The submerged spore wall averaged 22 nm in thickness, intermediate in size between the aerial spore wall (37 nm) and that of a mycelium (18 nm). No other major differences between the micrographs of submerged spores and aerial spores were found (Fig. 2).
TABLE 1. List of S. griseus strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-2682</td>
<td>Wild type</td>
<td>NRRL</td>
</tr>
<tr>
<td>SKK809</td>
<td>Nic&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B-2682, FUV&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SKK813</td>
<td>Trp&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B-2682, FUV</td>
</tr>
<tr>
<td>SKK815</td>
<td>Ser&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B-2682, FUV</td>
</tr>
<tr>
<td>SKK817</td>
<td>Bld&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B-2682, FUV</td>
</tr>
<tr>
<td>SKK820</td>
<td>Ths&lt;sup&gt;d&lt;/sup&gt;</td>
<td>B-2682, spontaneous</td>
</tr>
</tbody>
</table>

<sup>a</sup> NRRL, Northern Regional Research Laboratory.
<sup>b</sup> FUV refers to mutagenesis by UV light at the median wavelength of 260 nm.
<sup>c</sup> Bld<sup>c</sup> is characterized by the absence of aerial mycelia and spores.
<sup>d</sup> Ths<sup>d</sup> refers to thiostrepton resistance.

We also compared the two spore types with respect to total spore protein, using two-dimensional polyacrylamide gel electrophoresis. Most of the proteins from the aerial-spore sample had counterparts in the submerged-spore sample; however, some proteins were unique to aerial spores.

Figure 3 indicates that both spore types formed germ tubes. In both cases, germination occurred more rapidly after heat activation at 45°C for 15 min.

**Nutritional aspects of sporulation.** Our initial nutritional studies indicated that sporulation of strain B-2682 occurred after growth in all media containing single carbon and nitrogen sources. Sporulation did not occur in cultures grown in a rich medium, such as phosphate-buffered medium C plus 10 g of peptone per liter (Table 3). We therefore investigated the time course of the life cycle of strain B-2682 growing in the defined medium with single carbon and nitrogen sources (Fig. 4). Under conditions in which the initial concentration of glucose was

![Image of life cycle](http://jb.asm.org/)
high, and that of ammonia was low (Fig. 4A), growth proceeded until 30 h, when all of the ammonia was utilized. At this point, the number of spores began to increase, reaching a maximum at 50 h. The glucose concentration decreased slowly during the incubation period. In another experiment, in which the initial concentration of glucose was low, and that of ammonia was high, an increase in spore concentration was not detected until after growth had stopped (Fig. 4B). The pH of both cultures remained between 7.2 and 7.6. No extracellular protease activity was detected, nor was any streptomycin evident in the culture supernatant under these experimental conditions (data not shown).

The preceding experiments suggested that either carbon or nitrogen limitation results in the sporulation of _S. griseus_. A major disadvantage to this study was that the point of initiation of sporulation could not be ascertained. We therefore turned to nutritional downshift studies to determine (i) whether sporulation could be induced in streptomycetes by incubation under starvation conditions and (ii) the time course of sporulation.

The morphological changes associated with sporulation after ammonia or phosphate depletion closely paralleled those observed during sporulation of the organism in complete medium (Fig. 1). A phosphate-depleted culture existed as typical vegetative mycelia through the first 4 h after downshift. At 4 h, the mycelial tips were slightly swollen. This swelling was accentuated through 8 h, when the swollen zones were elongated and bulging. By 12 h, free spores were evident, as were spore chains. The entire process was similar, but less pronounced, and was delayed for approximately 4 h in the ammonia-

### TABLE 2. Properties of submerged and aerial spores and mycelia of _S. griseus_

<table>
<thead>
<tr>
<th>S. griseus structure</th>
<th>Trehalose (dry wt)</th>
<th>Endogenous respiration [O(2)]*</th>
<th>Germination</th>
<th>Sonic resistance</th>
<th>Lysozyme resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submerged</td>
<td>2.7 (0.0)c</td>
<td>9.5 (2.5)</td>
<td>70</td>
<td>75</td>
<td>2.0 (0.85)</td>
</tr>
<tr>
<td>Aerial</td>
<td>10.2 (4.5)</td>
<td>4.5 (0.4)</td>
<td>90</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>Mycelia</td>
<td>&lt;0.08</td>
<td>83.6 (13.5)</td>
<td>NAf</td>
<td>&lt;0.03</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Oxygen quotient (microliters of O₂ per hour per milligram [dry weight]) in 50 mM K-PO₄, pH 7.2.

* Percent germ tube formation after heat activation at 45°C for 15 min and subsequent incubation for 4 h at 32°C in yeast extract (1 g/liter)-50 mM K-PO₄, pH 7.2.

* Percent survivors of 10 min of sonication.

* Percent survivors of 1 h of incubation at 37°C in the presence of 20 μg of lysozyme per ml.

* The values in parentheses indicate the standard deviations.

* NA, Not applicable.
depleted culture. These results are depicted graphically in Fig. 5. After ammonia depletion, the culture underwent a slight increase in turbidity before the appearance of free spores. The increase in sonic resistance preceded the microscopic appearance of spores by approximately 2 h. The ammonia concentration in the culture fluid was less than 1 μM throughout the experiment, whereas the phosphate concentration remained at approximately 35 mM. The glucose concentration fell gradually, reaching 15 mM by 32 h.

The phosphate-depleted culture (Fig. 5) showed a dramatic increase in turbidity. This
depicted graphically in Fig. 5. After ammonia depletion, the culture underwent a slight increase in turbidity before the appearance of free spores. The increase in sonic resistance preceded the microscopic appearance of spores by approximately 2 h. The ammonia concentration in the culture fluid was less than 1 μM throughout the experiment, whereas the phosphate concentration remained at approximately 35 mM. The glucose concentration fell gradually, reaching 15 mM by 32 h.

The phosphate-depleted culture (Fig. 5) showed a dramatic increase in turbidity. This
Commitment to sporulate. Because strain B-2682 did not sporulate in rich medium, we could determine whether an induced culture reaches a critical point, beyond which the culture sporulates in spite of exposure to inhibitory conditions. We therefore subjected either phosphate- or ammonia-depleted cultures to nutrient-rich conditions at various times after nutrient depletion. The cultures were incubated for a total of 30 h, at which point the sonic-resistant units per milliliter was determined for each sample. The results (Table 4) indicate that ammonia-depleted cultures became committed to sonic resistance by 9 h after downshift. The phosphate-depleted culture behaved similarly, reaching commitment by 6 h after downshift. In both experiments, microscopic examination at 30 h indicated that free spores were present in all cultures that showed an increase in the sonic-resistant units per milliliter.

Enzyme activities during sporulation. One of our goals in devising a sporulation system was to identify either a sporulation-specific enzyme or an enzyme activity that fluctuated characteristicly during the transformation from a vegetative to a sporulating culture. In preliminary experiments, we observed that the activities of glutamine synthetase, alkaline phosphatase, and glucose-6-phosphate dehydrogenase fluctuated at various stages of the life cycle. We therefore assayed these enzymes in cultures subjected to nutritional downshift. Under ammonia starvation conditions, alkaline phosphatase activity remained at the low initial level, whereas the specific activity of glutamine synthetase increased at a rate of 0.23 h⁻¹ until reaching a plateau at 9 h (data not shown). During phosphate limitation (Fig. 6), alkaline phosphatase activity did not increase until at least 12 h after...

**FIG. 5.** Culture parameters after nutritional downshift of *S. griseus* B-2682. (A) Growth (Klett units) and spores (sonic-resistant units [SRU]) during either ammonia or phosphate deprivation are shown; (B) exogenous glucose and inorganic phosphate concentrations after ammonia deprivation are given; (C) exogenous glucose and ammonia concentrations after phosphate deprivation are given.

was due primarily to an increase in dry weight, but also to the development of refractile spores (data not shown). Again, the increase in sonic resistance slightly preceded the appearance of spores. The culture supernatant contained less than 1 μM inorganic phosphate throughout the experiment. The ammonia concentration decreased to 16 mM, and the glucose concentration decreased to 12 mM, by 32 h, at which point spores constituted more than 90% of the culture.

When depleted for glucose (data not shown), at least 90% of the mycelia lysed within 20 h. The remaining mycelia did eventually sporulate.

The ability of both ammonia- and phosphate-starved cultures to sporulate suggested that a significant amount of the sporulation-specific metabolism utilized endogenous precursors. This hypothesis was tested by subjecting a tryptophan auxotroph (SKK813) to starvation conditions. The results (Table 3) indicate that there was insufficient intracellular tryptophan to support the sporulation of this strain. A serine auxotroph (SKK815), however, contained sufficient amounts of glycine or serine to effect sporulation in the absence of either amino acid in the phosphate-depleted medium (Table 3).

**TABLE 4.** Time required for commitment of *S. griseus* B-2682 to sporulate after ammonia or phosphate depletion

<table>
<thead>
<tr>
<th>Time (h) before nutrient replenishment</th>
<th>Ammonia-starved culture</th>
<th>Phosphate-starved culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.01</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>0.01</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>0.17</td>
</tr>
<tr>
<td>8</td>
<td>0.06</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>0.29</td>
<td>0.23</td>
</tr>
<tr>
<td>12</td>
<td>0.17</td>
<td>0.37</td>
</tr>
<tr>
<td>30</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Sonic-resistant units (SRU) at 30 h relative to the sample that was starved for 30 h.

ND, Not determined.
downshift. Under these same conditions, glutamine synthetase activity displayed an initial rapid increase, followed by an intermediate decrease. By 30 h, glutamine synthetase activity had regained its high level. No glucose-6-phosphate dehydrogenase activity could be detected during either starvation period, although this enzyme was active in cultures growing exponentially with glucose as the sole source of carbon.

**Mutant studies.** Several thiostrepton-, rifampin-, or neomycin-resistant spontaneous mutants were isolated. None of these mutants showed any defect in sporulation on solid media, in the presence or absence of the drug, and at high (37°C) or low (28°C) temperature. The thiostrepton-resistant strain SKK820 was unimpaired in its sporulation properties after nutritional downshift in the presence of 1 µg of thiostrepton per ml, whether ammonia or phosphate depleted (Table 3).

Most of the auxotrophs that were isolated after UV mutagenesis (e.g., Trp<sup>−</sup> Ade<sup>−</sup>, Met<sup>−</sup>) sporulated normally. However, a strain that required nicotinamide (SKK809) and one that required either serine or glycine (SKK815) sporulated poorly on medium A or on supplemented medium B. Neither of these strains excreted streptomycin under conditions in which the wild-type strain produced approximately 7 µg/ml. An antibiotic inhibitory to both streptomycin-resistant and streptomycin-sensitive indicator strains was produced by both auxotrophs. The serine auxotroph reverted to serine independence at a frequency of $3 \times 10^{-6}$ and simultaneously regained the ability to sporulate well on medium B. Strain SKK809 reverted to nicotinamide independence at a frequency of $2.4 \times 10^{-4}$; of these revertants, 3% sporulated normally.

One bald mutant, strain SKK817, defective in aerial spore formation, was isolated after UV mutagenesis. This strain reverted to normal sporulation at a frequency of less than $1 \times 10^{-9}$. Although strain SKK817 produced no streptomycin, at least one substance inhibitory to both indicator strains was excreted. The sensitivity of strain SKK817 to streptomycin (inhibition by more than 10 µg of streptomycin sulfate per ml) was identical to that of the wild-type strain. Strain SKK817 did not sporulate on medium A or in medium C containing various carbon and nitrogen sources. Ammonia depletion conditions resulted in a sporulation efficiency of less than 0.07 (Table 3). The wild-type strain of *S. griseus* did not excrete any substance that induced strain SKK817 to sporulate in either solid or liquid culture, nor did strain SKK817 produce an agent that inhibited the sporulation of strain B-2682.

**DISCUSSION**

An analysis of a variety of spore properties indicates that the submerged spores of *S. griseus* B-2682 are similar, but not identical, to aerial spores. Both submerged and aerial spores contain relatively high levels of trehalose, a sugar virtually absent from exponentially growing mycelia. The endogenous respiration rate of each spore type was considerably lower than that of actively respiring mycelia, consistent with the conclusion that mature streptomycete spores are physiologically dormant (8). The apparent increased oxygen quotient of submerged spores relative to aerial spores may be accounted for by a 5% level of contamination by mycelia in the submerged spore preparation. The germination of both submerged and aerial spores responded to heat activation. The major difference between submerged and aerial spores was the marked sensitivity of the former to lysozyme treatment. This sensitivity appears to be independent of the presence of a sheath surrounding the spore chain, since we were unable to observe the sheath consistently on either spore type. The sensitivity to lysozyme may result from the thinness of the submerged spore wall.

Our initial studies of the life cycle suggested that either nitrogen or carbon depletion may elicit sporulation. It is clear that sporulation occurs independently of streptomycin excretion, extracellular protease activity, and changes in the pH of the medium. The nutritional downshift studies indicate that nitrogen or phosphate deprivation is an effective nutritional trigger of sporulation. Phosphate depletion studies suggest
that vegetative mycelia possess a large pool of phosphate available to the sporulating organism.

Although we have been unable to identify a sporulation-specific enzyme the appearance of whose activity signals the initiation of sporulation, the reproducible fluctuation in glutamine synthetase activity suggests that this enzyme may be useful as a sporulation marker under phosphate depletion conditions. The rapid increase in glutamine synthetase activity after phosphate deprivation is striking in light of the observation that glutamine synthetase from *Streptomyces cattleya* is adenyllylated and, thereby, inactivated in response to a high concentration of ammonia (21). The continued low activity of alkaline phosphatase in the absence of exogenous phosphate supports the hypothesis that mycelia contain large pools of inorganic phosphate; these pools apparently must be depleted before alkaline phosphatase activity can increase.

Based on results obtained with *Bacillus* strains (12, 14), we predicted that drugs that interfere with transcription or translation in streptomycetes may select for resistant mutants that concomitantly have acquired altered sporulation properties. Although our search for such mutants was not extensive, our initial results do not substantiate this hypothesis. Chater similarly reported that several rifampin-resistant mutants of *Streptomyces coelicolor* did not simultaneously become defective in differentiation (2).

Among the auxotrophic mutants isolated were two that were oligosporogenous, sporulating poorly on media that ordinarily supported abundant sporulation. Reversion analysis of the serine auxotroph indicated that a single, revertible mutation was responsible for both the serine requirement and the oligosporogeneity; likewise, a single mutation appears to have resulted in both nicotinamide auxotrophy and poor sporulation of strain SKK809. That strain SKK815 does sporulate when subjected to phosphate starvation indicates that this strain is a conditionally sporulating mutant. Although data mentioned earlier suggest that streptomycin excretion is not obligatory for sporulation, the fact that neither the serine auxotroph nor the nicotinamide auxotroph produced extracellular streptomycin suggests that the biosynthesis of the antibiotic may be indirectly correlated with the ability to form spores, as other investigators have suggested (4, 19). This hypothesis is also supported by the inability of strain SKK817 to produce streptomycin. In all cases examined, at least one bioactive substance was produced by strains that did not sporulate, indicating that the correlation between sporulation and antibiotic excretion may be specific for streptomycin.

Unlike strain SKK815, which can sporulate under certain culture conditions, strain SKK817 appears to be asporogenous under all conditions. Phase-contrast microscopy revealed no spores when strain SKK817 was subjected to nutritional downshift. We presume that the measured SE (Table 3) reflects a limited number of mycelial fragments that escaped sonic disruption. Our inability to detect normal sporulation revertants of strain SKK817 suggests that a deletion or gene rearrangement is responsible for the pleiotropic phenotype of this strain. Bíró and colleagues (1) and Khokhlov et al. (11) have reported the production, by sporulating strains of *S. griseus*, of two potent factors, each of which promotes the sporulation of certain bald mutants. Our results indicate that strain SKK817 does not sporulate in response to exposure to culture fluid from the wild-type strain. Parallel experiments show that spent culture medium from strain SKK817 does not inhibit the sporulation of strain B-2682.

The results in this paper demonstrate that *S. griseus* B-2682 can sporulate abundantly in a liquid culture under appropriate nutritional and environmental conditions. We have observed that other strains of *S. griseus* can also sporulate in a submerged culture and that *S. viridochromogenes* can be induced to sporulate by nitrogen starvation in a liquid medium (R. Koepsel and J. C. Ensign, manuscript in preparation). After nitrogen or phosphate deprivation, *S. griseus* rapidly sporulates. Additionally, C. Hirsch and L. Koupal have observed that an unknown streptomycete soil isolate sporulates well in a defined, phosphate-limited medium (personal communication). Streptomycete sporulation may, therefore, be a feasible model system for the study of the physiological and molecular events involved in differentiation, events that were previously difficult to study with cultures growing on solid media.

**ACKNOWLEDGMENTS**

This work was supported by NRSA postdoctoral training grant F32GM07405 to K.E.K. from the National Institutes of Health.

We thank Martin Garment for the electron microscopy.

**LITERATURE CITED**


